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Identification of citrus immune regulators involved in defence against Huanglongbing using a new functional screening system

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Summary

Huanglongbing (HLB) is the most devastating citrus disease in the world. Almost all commercial citrus varieties are susceptible to the causal bacterium, *Candidatus* Liberibacter asiaticus (CLas), which is transmitted by the Asian citrus psyllid (ACP). Currently, there are no effective management strategies to control HLB. HLB-tolerant traits have been reported in some citrus relatives and citrus hybrids, which offer a direct pathway for discovering natural defence regulators to combat HLB. Through comparative analysis of small RNA profiles and target gene expression between an HLB-tolerant citrus hybrid (*Poncirus trifoliata* × *Citrus reticulata*) and a susceptible citrus variety, we identified a panel of candidate defence regulators for HLB-tolerance. These regulators display similar expression patterns in another HLB-tolerant citrus relative, with a distinct genetic and geographic background, the Sydney hybrid (*Microcitrus virgata*). Because the functional validation of candidate regulators in tree crops is always challenging, we developed a novel rapid functional screening method, using a *C.* Liberibacter solanacearum (CLso)/potato psyllid/*Nicotiana benthamiana* interaction system to mimic the natural transmission and infection circuit of the HLB complex. When combined with efficient virus-induced gene silencing in *N. benthamiana*, this innovative and cost-effective screening method allows for rapid identification and functional characterization of regulators involved in plant immune responses against HLB, such as the positive regulator BRCA1-Associated Protein, and the negative regulator Vascular Associated Death Protein.

Introduction

Huanglongbing (HLB), also known as citrus greening disease, is the most destructive disease threatening the global citrus industry. Since the first US case of HLB was discovered in Florida in 2005 (Stokstad, 2012), the disease has spread to most citrus production states, including Texas and California. Despite extensive efforts and research, there are no effective control strategies for the disease. HLB is caused by a fastidious bacterium *Candidatus* Liberibacter asiaticus (CLas), which is transmitted by the insect vector Asian citrus psyllid (ACP) *Diaphorina citri* (Bove, 2006). Along with drastic losses in fruit production, the need to increase insecticide applications for vector control has raised costs significantly, making citrus production for growers very difficult in affected areas. The current lack of methods to effectively combat HLB makes discovery of innovative control strategies imperative for the survival of the citrus industry.

Almost all citrus species including most commercial varieties are susceptible to CLas, with the exception of a few tolerant citrus relatives, such as *Poncirus trifoliata* L. Raf. and some of its hybrids (Albrecht and Bowman, 2011, 2012a), which are commonly used as rootstocks. These HLB-tolerant varieties are a valuable resource for the discovery of the natural important regulators involved in defence against HLB. US-942, a hybrid of Sunki mandarin (*Citrus reticulata*) and trifoliate orange (*Poncirus trifoliata)*, is one of the most popular HLB-tolerant rootstocks in Florida because it can induce production of good yield and fruit quality of commercial scions under HLB-endemic conditions (Bowman et al., 2016). However, the functional regulators linked to the HLB-tolerant phenotype of US-942 are still unclear (Albrecht and Bowman, 2012a,b; Albrecht *et al*., 2016).

Plant endogenous small RNAs (sRNAs), including both micro-RNAs (miRNAs) and small interfering RNAs (siRNAs), have emerged as important regulators of gene expression in plant immune responses against pathogen infection (Cai *et al*., 2018;

Huang *et al*., 2019; Li *et al*., 2017). Bacterial infection can lead to up- or down-regulation of sRNAs, which then suppress or de-suppress expression of their target genes, contributing to plant defence (Katiyar-Agarwal *et al*., 2007; Katiyar-Agarwal *et al*., 2006; Navarro *et al*., 2006; Zhang *et al*., 2011). sRNA-mediated regulatory mechanisms have also been identified in crop plants by characterizing host sRNA profiles during pathogen attack (Huang *et al*., 2019). In the citrus-*C*Las pathosystem specifically, a high accumulation of the miR399 in *C*Las-infected trees revealed a link between phosphate starvation and HLB response (Zhao *et al*., 2013). Here, we report that sRNA profiling is a powerful tool for identifying key regulators of citrus defence responses against *C*Las. Through comparative analysis of sRNA profiles of the HLB-tolerant hybrid, US-942 and the HLB-susceptible Cleopatra mandarin, we have identified panels of sRNAs and their targets with unique response patterns to *C*Las in the US-942 background and revealed potential important regulators contributing to the HLB-tolerant phenotype. The expression patterns of these candidate regulators in the tolerant US-942 background are similar to those in the genetically distinct and geographically separated HLB-tolerant citrus relative, Sydney hybrid (*Microcitrus virgata*), further supporting that the genes identified in the current study are important regulators associated with HLB-tolerance.

Thus far, functional studies of HLB-tolerance related genes have been hampered by the inability to obtain a pure culture of *C*Las, the significant time and effort required for genetic study of gene functions in citrus trees, and the vector-mediated transmission nature of the pathogen. A rapid large-scale functional screening method for host immune regulators that contribute to defence against *C*. Liberibacter species is urgently needed. To fill this need, we developed an effective host/vector/pathogen interaction system using a close relative of *C*Las, *C*. Liberibacter solanacearum (*C*Lso), which infects solanaceous plants, the potato psyllid, a major pest of potatoes and tomatoes, and *Nicotiana benthamiana*, the ideal hosts for virus-induced gene silencing (VIGS) experiments. VIGS is an effective silencing method to knock down expression of plant endogenous genes using a viral vector. This system is very similar to the natural citrus/psyllid/CLas interaction system and can be used to rapidly characterize the function of candidate regulators in plant defence responses against *C*. Liberibacter species.

Results

A set of miRNAs and siRNAs is more abundant in the tolerant hybrid US-942

Through comparing the sRNA profiles of uninfected HLB-tolerant hybrid US-942 and uninfected HLB-sensitive mandarin Cleopatra, we discovered 19 conserved miRNAs which are constitutively more abundant in US-942 than in the HLB-susceptible Cleopatra when using >5 reads per million total normalized reads (rpm) and >3 fold change as cutoffs (Table S1). Three miRNAs are constitutively less abundant in US-942 than in Cleopatra (Table S1). MiR399 has a much higher constitutive expression level in US-942 than in Cleopatra and is further induced upon *C*Las infection (Figure 1a). Our previous study discovered that miR399 expression is highly induced after *C*Las infection in most HLB-susceptible citrus varieties (Zhao *et al*., 2013). In *Arabidopsis*, miR399 expression is induced by P_i-deficiency and plays a role in the regulation of P_i homoeostasis (Hsieh *et al*., 2009). Previously, we demonstrated that HLB-positive citrus trees contain significantly lower levels of phosphorus (P_i) than uninfected trees and reported this as one of the major causes for HLB disease symptoms (Zhao *et al*., 2013). MiR399 targets and silences an E2 ubiquitin conjugating enzyme *(PHO2)* that is responsible for the degradation of P_i transporters, which results in increased P_i transporters and P_i uptake (Zhao *et al*., 2013). Here, we show that the target gene of miR399, *PHO2*, has significantly lower expression in US-942 compared to Cleopatra with or without *C*Las infection (Figure 1b,c). Additionally, the two P_i transporters, PT2 and PHT2, both have higher expression levels in US-942 than in Cleopatra (Figure 1b,c). Thus, gene regulation mediated by enhanced expression of miR399 makes US-942 better equipped to cope with P_i starvation after *C*Las infection.

To probe whether siRNAs of US-942 are also expressed differentially from Cleopatra, we first focused on siRNAs which have differential constitutive expression in US-942 in comparison to Cleopatra. To do this, we compared the siRNA profiles of uninfected US-942 and Cleopatra and identified a panel of siRNAs with 3-fold higher or lower expression in US-942 than in Cleopatra. 209 siRNAs have higher constitutive expression levels in US-942, which remain high upon *C*Las infection (Table S2). In order to determine what proportion of these sRNAs were contributed to US-942 by the *P. trifoliata* genome, we searched these 209 sRNA sequences in the *P. trifoliata* genome database. We found that 49% of these sRNAs (103 out of 209 sRNAs) could be encoded by *P. trifoliata* genome (Table S2). *In silico* target prediction of identified siRNAs was performed against the transcript database of *Citrus clementina*, the closest relative to US-942 with an available sequenced genome, using the base pairing prediction score ≤4 as a cutoff (Dai *et al*., 2018). The target genes of these siRNAs would be silenced to a greater degree in US-942 than in Cleopatra and could play a negative role in natural defence against HLB (Table S3). Further, we identified 293 sRNAs with 3-fold lower expression in US-942 than in Cleopatra, which remain low after *C*Las infection (Table S4). Among these 293 sRNAs, 48%, 140 sRNAs, are represented in the *P. trifoliata* genome (Table S4). It is likely that these siRNAs silence target genes which contribute positively to plant defence responses (Table S5). Indeed, a Gene Ontology (GO) enrichment analysis demonstrated that the predicted target genes are enriched in GO terms relating to defence response to bacterium and callose deposition (da Huang *et al*., 2009) (Figure S1a). Lower expression levels of these siRNAs would lead to higher expression of these putative positive regulators, making US-942 better equipped to defend against HLB.

Identification of important defence regulators from US-942

To identify natural plant defence regulators in response to *C*Las infection, we focused on the sRNAs which displayed differential expression patterns both before and after *C*Las infection only in the HLB-tolerant hybrid US-942, because these sRNAs and their target genes likely contribute to HLB-tolerance. Regulators with both a rapid (within two months post *C*Las infection) and extended response (six months post *C*Las infection) are more likely to provide strong and durable protection from HLB than genes with transient regulatory patterns. Leaf samples from US-942 and Cleopatra at 7 and 28 weeks post grafting inoculation (wpi) of *C*Las were subjected to sRNA profiling, with uninfected leaves used as controls. The sRNAs with similar response patterns in both US-942 and Cleopatra were eliminated from analysis. A total of 371 sRNAs were up-regulated more than 3-fold at both 7 and 28 wpi in US-942, but not in Cleopatra (Table S6), and 100

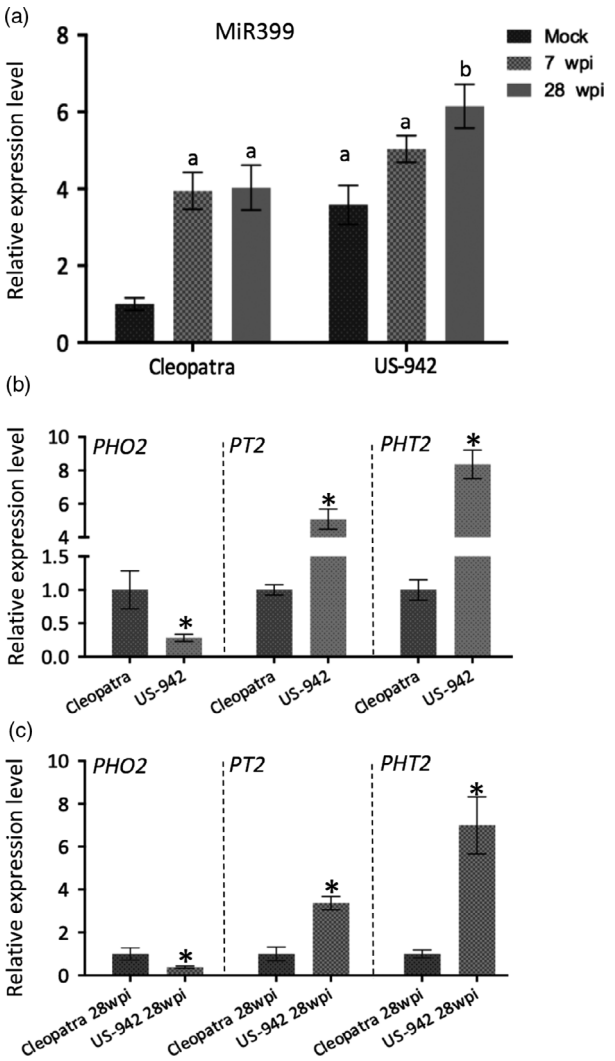

Figure 1 HLB-tolerant US-942 has a higher constitutive expression level of miR399 than Cleopatra. (a) Expression levels of miR399 were detected by qRT-PCR in infected Cleopatra and US-942 plants at 7 and 28 weeks post inoculation (wpi) and compared to healthy plants (Mock). Actin was used as the reference gene. Data are shown as means ± SE. Different letters indicate a significant difference between groups ($P < 0.05$; determined by one-way ANOVA by Tukey's multiple comparison tests). (b, c) Expression levels of miR399 target gene *PHO2*, and phosphate transporter *PT2* and *PHT2* detected by qRT-PCR in Cleopatra and US-942 without infection (b) and with infection (c). Actin was used as the reference gene. * indicates a significant difference ($P < 0.01$; determined by Student's *t*-test).

sRNAs were down-regulated by more than 3-fold only in US-942 (Table S7). Among the 371 up-regulated sRNAs and the 100 down-regulated sRNAs, 30% and 11% were present in the *P. trifoliata* genome, respectively (Table S6 and S7). We selected the top fifty sRNAs from both lists and performed *in silico* target prediction analysis using a stringent base pairing prediction score of ≤4 as a cutoff (Table S8 and S9). These sRNA targets were enriched in the GO terms of plant defence responses, induced systemic resistance response, and hypersensitive response (Figure S1b). These genes have a high likelihood of contributing to HLB-tolerance for US-942. The target genes of the down-regulated sRNAs are potentially upregulated at the transcriptional or translational level and act as positive regulators of host defence responses against HLB (Table S9). Conversely, the target genes of upregulated sRNAs are likely to be negative regulators of plant defence genes responding to *C*Las infection, which are suppressed by these sRNAs (Table S8).

Candidate defence regulators identified from US-942 show similar expression patterns in another HLB-tolerant citrus relative

To identify potential regulators that contribute to HLB tolerance, we selected the sRNA target genes with known or predicted functions in plant defence responses for further functional analysis (Figure 2a). The expression level of the sRNAs and their target genes in *C*Las-infected US-942 and Cleopatra was validated by quantitative RT-PCR (Figure 2b–e, S3 and S4). Two sRNAs specifically down-regulated in US-942 after *C*Las infection, 942si1001 and 942si1020 (Table S7), were experimentally confirmed to have lower expression levels in US-942. The two sRNAs specifically up-regulated in US-942 after *C*Las infection, 942si2003 and 942si2048 (Table S6), were confirmed to have a higher expression level in US-942 (Figure 2b). In all four cases, target gene expression is clearly negatively correlated with the expression of corresponding sRNAs. The targets of 942si1001 and 942si1020, BRCA1-Associated Protein (*BRAP)* and cytochrome P450 *CYP93*, respectively, had 3-4 fold higher transcription levels in US-942 than in Cleopatra (Figure 2d). *BRAP* is an E3-ligase which positively regulates pathogen-associated molecular pattern triggered defence response in plants and *CYP93s* are involved in the synthesis of a phytoalexin in soybean (Park *et al*., 2016; Schopfer *et al*., 1998). It is likely that both *BRAP* and *CYP93* positively regulate plant defence and are upregulated in response to the down-regulation of their corresponding siRNAs during *C*Las infection in US-942. The targets of 942si2003 and 942si2048, Oligopeptide Transporter 1 *(OPT1)* and Vascular Associated Death *(VAD)*, respectively, displayed expected negative expression correlation to their corresponding sRNAs (Figure 2b,d). In *Arabidopsis*, the oligopeptide transporter family mediates the uptake of a virulence factor, Syringolin A, which is secreted by plant pathogen *Pseudomonas syringae pv. syringae* (Hofstetter *et al*., 2013). Further, the *vad1 Arabidopsis* plant is resistant to bacterial pathogens due to increased salicylic acid accumulation (Lorrain *et al*., 2004). Thus, citrus OPT1 and VAD1 likely act as negative regulators of plant defence, which are silenced by sRNAs after *C*Las infection in US-942.

We also examined the expression patterns of these selected candidate regulators in an HLB-tolerant citrus relative, Sydney hybrid (*Microcitrus virgata* or *Citrus virgata*), a hybrid of the Australian round lime, *Microcitrus australis* and the Australian finger lime, *M. australasica* (Reuther, *et al*., 1967). The progenies of the Sydney hybrid display segregation of the HLB-tolerance trait (Ramadugu *et al*., 2016). Among them, Sydney72 (Syd72) shows strong tolerance to HLB, whereas Syd74 is highly susceptible to *C*Las infection. Similar to US-942, Syd72 has higher expression levels of miR399 compared to Syd74 (Figure S2). We further demonstrate that these identified sRNAs (Figure 2b,c) and their target regulators (Figure 2d,e) displayed similar expression patterns in both HLB-tolerant hybrids, Syd72 and US-942. These results further support that these selected plant defence regulators are possibly involved in HLB-tolerance responses because they display the same unique expression pattern in both HLB-tolerant hybrids.

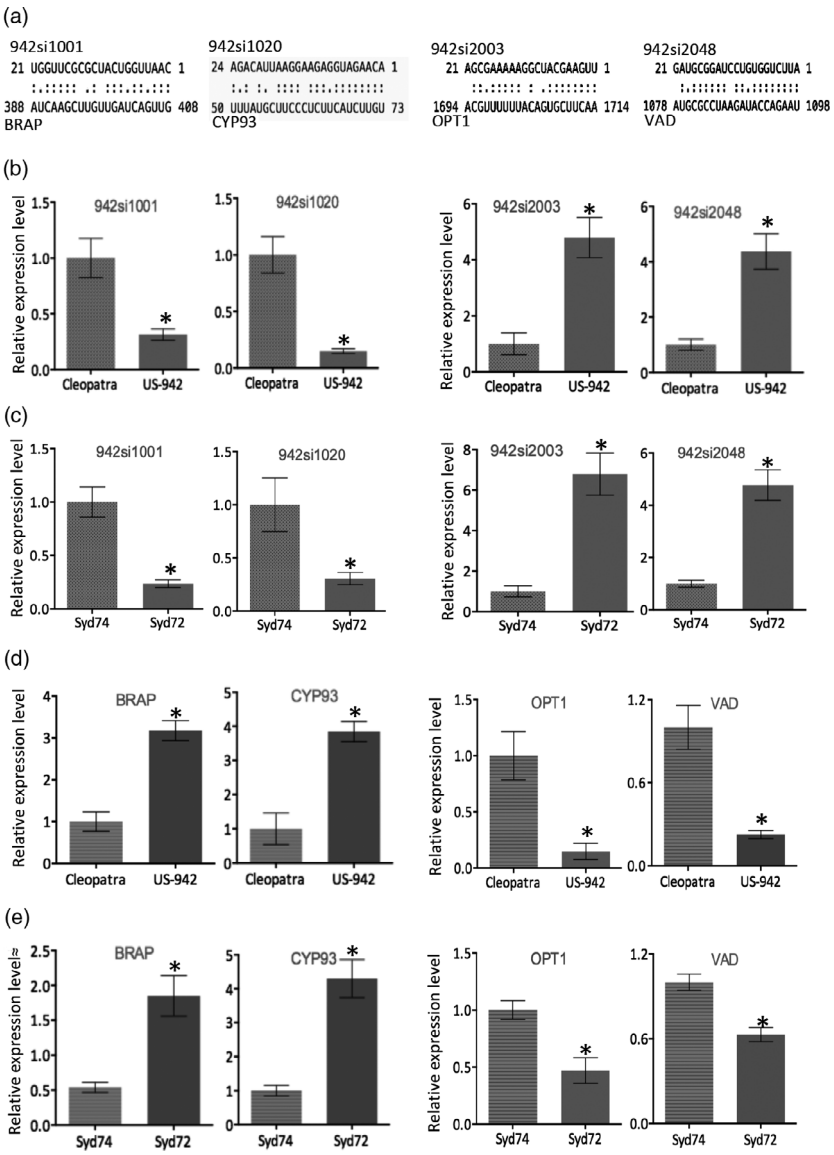

Figure 2 Four selected defence regulators with potential contributions to HLB-tolerance have similar expression levels in two genetically distinct hybrid varieties. (a) The target prediction of four sRNAs potentially contributing to HLB-tolerance. (b, c) Verification of the expression levels of four sRNAs (panel a) in two sets of genetically distinct and geographically separated citrus and citrus relatives, US-942 versus Cleopatra (28 wpi) (b) and Sydney 72 versus Sydney 74 (c), after *C*Las infection. Expression levels were detected by qRT-PCR with Actin as the reference gene. * indicates a significant difference ($P < 0.05$; determined by Student's *t*-test). (d, e) Verification of the expression levels of target genes of four sRNAs in US-942 versus Cleopatra (28 wpi) (d) and Sydney 72 versus Sydney 74 (e), after *C*Las infection. Expression levels were detected by qRT-PCR with Actin as the reference gene. * indicates a significant difference ($P < 0.05$; determined by Student's *t*-test).

Establishment of a new *Nb*/Potato psyllid/*CLso* pathosystem that is similar to the citrus/Asian citrus psyllid/CLas pathosystem

Functional characterization of genes, protectants or therapeutic treatments on citrus has been difficult due to the extended lifespan of citrus trees and the fluctuating patterns of HLB development within a tree (Fletcher *et al*., 2018). Further, genetic manipulation of citrus requires significant time and effort, which makes large-scale functional genetic screening almost impossible. To validate and rapidly screen the functions of potential regulators, identified through sRNA profiling and gene expression analysis, in HLB defence response, we developed a novel screening system by infecting the model plant, *Nicotiana benthamiana* (*Nb*), with *C*Lso through transmission by the potato psyllid. This pathosystem can serve as a surrogate for the citrus/ACP/CLas pathosystem and allow rapid large-scale functional screening of candidate genes and other molecules. We chose to use *Nb* because it is an ideal host for efficient virus-induced gene silencing (VIGS; Ratcliff *et al*., 2001), which allows for rapid determination of gene function by knocking down gene expression using a viral expression vector. Although *Nb* is not a natural host for the potato psyllid and *C*Lso, we discovered that potato psyllid nymphs can feed on very young *Nb* plants. Two-week-old *Nb* were exposed to *C*Lso-positive potato psyllid nymphs for 5 days (Figure 3a). Three weeks later, there was observable yellowing symptoms and vascular tissue greening in leaves (Figure 3b,c). Additionally, the *C*Lso 16S ribosomal RNA gene was detected by PCR (Figure 3d) in leaves that were directly fed upon as well as those systemic leaves. During feeding, psyllid nymphs and possibly adults, inject toxic saliva into plant foliage and cause feeding damage known as 'psyllid yellows' (Liu and Trumble, 2004). The 'psyllid yellow' symptom was observed on *Nb* plants exposed to both *C*Lso-positive and *C*Lso-negative psyllids 5 days after feeding (Figure S5). *Nb* plants fed to *C*Lso-negative psyllids recovered from the damage caused by psyllid feeding, whereas plants fed to *C*Lso-positive psyllids developed further chlorosis symptoms (Figure 3e and S5). The *C*Lso bacterial titre was detected by probe-based qPCR. At three wpi, the bacterial titre ranged from 10^5 to 10^8 copies per 50 ng plant

genomic DNA whereas no bacteria were detected in plants exposed to *C*Lso-negative psyllids (Figure 3f).

This model system allows us to detect *C*Lso infection as early as three wpi. This is a significant advantage over the current system where six to 12 months may be required from the inoculation of *C*Las to the detection of bacteria and the development of disease symptoms in citrus (Fletcher *et al*., 2018). The *Nb*/Potato psyllid/*CLso* pathosystem we report here can provide results in 6-8 weeks, saving both time and resources.

Using VIGS and the *Nb*/Potato psyllid/*C*Lso pathosystem for rapid functional screening of potential regulators

A key advantage of the model *Nb*/psyllid/*CLso* pathosystem, in addition to shortening disease development time, is that the expression of target genes can be easily manipulated in *Nb* plants by VIGS (Ratcliff *et al*., 2001). *Tobacco rattle virus* (TRV) is the most widely used VIGS vector for gene functional analysis. TRV-based VIGS vectors have been used to silence genes in a number of Solanaceous plant species and are most effective in *Nb* (Liu *et al*., 2002; Ratcliff *et al*., 2001). To study the function of candidate regulators in natural defence responses against *C*Lso, we used TRV-based VIGS to knock-down the *Nb* orthologous/homologous genes of the candidate citrus regulators in *Nb* plants infected with *C*Lso.

The two-week-old *Nb* plants were exposed to *C*Lso-positive potato psyllid nymphs for 5 days. Three days after psyllid nymph removal, during which time the plants recovered from psyllid yellows, we performed TRV-based VIGS through *Agrobacterium tumefaciens* mediated leaf infiltration (Lu *et al*., 2003). The TRV construct containing a piece of *Solanum bulbocastanum*-specific late-blight resistance gene, RB (Song *et al*., 2003), was used as a negative control (siRB), as it has no target gene in *Nb*. As expected, *C*Lso disease formation and the gene knock-down effect both occurred within 2–3 weeks.

The screening results of the positive regulator, *BRAP*, and the negative regulator, *VAD* are shown in Figures 4 and 5. Compared to the negative control, knocking down *BRAP* led to a *C*Lso susceptible phenotype, as indicated by stunted growth and chlorosis (Figure 4a,b), supporting that BRAP acts as a positive regulator of plant defence responses against HLB. On the contrary, knocking down the negative regulator *VAD* gene, revealed a *C*Lso tolerant phenotype with weaker symptoms and, larger and healthier plants compared to the negative control (Figure 5a,b). Silencing efficiency of *BRAP* and *VAD* in all tested leaves was monitored by qRT-PCR (Figures 4c and 5c) and compared to the siRB control plants. The bacterial titre in the mid-vein from the leaves used to monitor *BRAP* and *VAD* silencing levels was detected by probe-based qPCR. Compared to the siRB controls, a significantly higher bacterial titre was detected in siBRAP plants (Figure 4d) and a significantly lower bacterial titre was detected in the siVAD plants (Figure 5d). Thus, *BRAP* was confirmed to be a positive regulator in plant defence response to *C*Lso, and *VAD* a negative regulator.

Discussion

Growing disease-resistant or -tolerant varieties is the most eco-friendly and cost-effective strategy for disease management. In the absence of naturally resistant varieties, one of the most attractive long-term and sustainable means of disease control is to identify important regulatory molecules and regulatory pathways in disease-tolerant/resistant hybrids or closely related species, and to work in concert with these natural plant responses to improve plant productivity in an HLB-endemic environment. Currently, US-942 is one of the most popular HLB-tolerant rootstocks in Florida (Bowman and Joubert, 2020; Bowman *et al*., 2016; Castle, 2010) and can serve as a rich genetic source for identification of plant genes that play roles in HLB defence. Rootstocks conduct nutrients, phytohormones, sRNAs, small proteins and metabolites to the scion. Some are systemic signalling molecules and can directly or indirectly affect scion gene expression (Wang *et al*., 2017). Citrus rootstocks affect the scion in many other ways, including tree vigour, fruit size, fruit quality and yield (Benjamin *et al*., 2013; Castle, 2010). They also play an important role in plant tolerance to abiotic and biotic stress (Castle, 2010; Dutra de Souza *et al*., 2017; Laino *et al*., 2016). The use of a *C*Las resistant rootstock may impart a positive effect on the phloem microenvironment, possibly mitigating the negative effects of *C*Las on the scion. However, no reliable breeding markers for the selection of HLB-tolerant progeny are currently known. Molecular markers will significantly accelerate the identification of disease-resistant/tolerant varieties for breeding programs. The sRNAs and their target genes we identified as important regulators in this study are potentially linked to the HLB resistance/tolerance phenotype. These regulators can be developed as selection molecular markers for rootstock and scion breeding.

Comparing the sRNA profiles of HLB-susceptible and tolerant plants allowed us to reveal how the pathogen differentially affected the sRNA regulatory network in plants. From our sRNA profile analysis, it appears that US-942 has a stronger defence response to *C*Las infection. For example, it is likely that US-942 can take up P_i more efficiently due to the high expression of miR399, contributing to the HLB-tolerant phenotype, and potentially rendering the grafted tree more tolerant to nutrient depletion due to *C*Las infection. Of the other miRNAs more abundant in US-942 (Table S1), miR159 and miR166 are involved in multiple hormone pathways and trigger stronger anti-bacterial defence responses (Zhang *et al*., 2011). MiR159, miR167, miR396 and miR408 have been demonstrated to contribute to drought-resistance (Hajyzadeh *et al*., 2015; Reyes and Chua, 2007; Sunkar and Zhu, 2004), and miR166, miR396, miR408 and miR477 were shown to be responsive to cold stress (Lu and Huang, 2008; Zhou *et al*., 2008). Taken together, these clues from differentially expressed miRNAs suggest that US-942 has a higher tolerance for certain biotic and abiotic stresses and can more efficiently take up nutrients than Cleopatra. Our results reveal that the HLB-tolerance of US-942 may not only rely on a particular pathway or a single regulator, but a sum of regulators, which when combined lead to a stronger defence response, more efficient nutrient uptake and the enrichment of metabolic products that benefit the host or impair pathogen survival. The application of the regulators requires further molecular genetic approaches and functional analysis to confirm the HLB-tolerant phenotype.

The complete genome sequence of *C*Las shows that the pathogen is likely to be parasitic rather than pathogenic, with the disease symptoms more similar to nutrient depletion or metabolic disorder (Duan *et al*., 2009). Studies of the transcriptome, the proteome and the metabolome have revealed some potentially important pathways and responses that appear to play a role in host tolerance to HLB (Albrecht and Bowman, 2012b; Albrecht *et al*., 2016; Albrecht *et al*., 2020; Hung and Wang, 2018; Wang

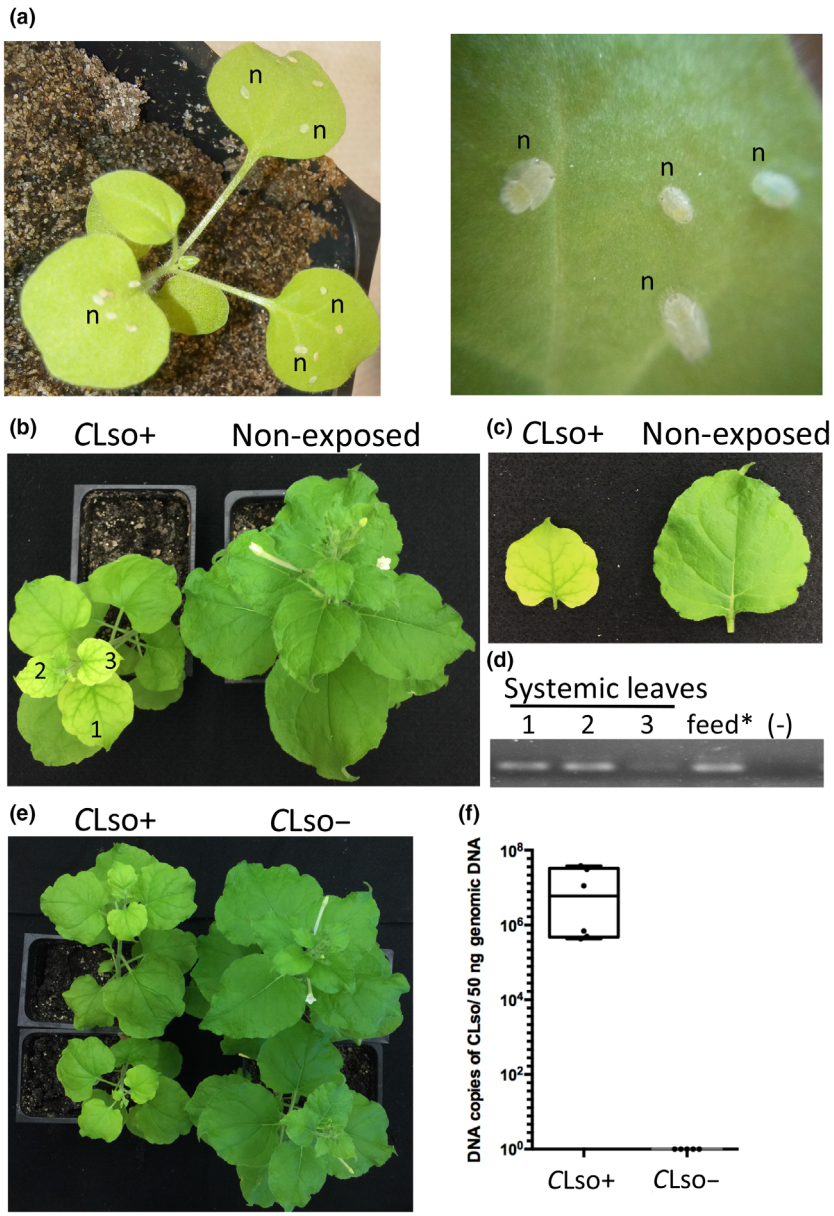

Figure 3 Establishing the *Nb*/Potato psyllid/CLso interaction as a surrogate pathosystem for the Citrus/Asian Citrus Psyllid/CLas pathosystem. (a) The 2 weeks old *Nb* plants were exposed to 15 potato pysllid nymphs (indicates as n) for 5 days. (b) *Nb* plants after exposure to CLso-positive pysllid nymphs (CLso+) for 5 weeks (left). The non-exposed plants are shown for comparison (right). (c) Leaf disease symptoms of *Nb* plant infected with CLso shown in panel b. (d) PCR detection of CLso in *Nb* plants in panel b. The 4 lanes show PCR products detected in 3 systemic leaves and the psyllid-exposed leaf (*) of a CLso-infected plant. The last lane shows the negative control (non-exposed plant). (e) *Nb* plants after exposure to CLso + and CLso-negative (CLso−) pysllid nymphs for three weeks. (f) The CLso bacterial titre detected by probe-based qPCR in 50 ng host genomic DNA of the plant tissue in panel e.

et al., 2016). In our sRNA profiling study, the results of a GO enrichment analysis also indicate that genes involved in metabolic pathways, such as flavonoid biosynthesis, are regulated by sRNAs and are differentially expressed in US-942, which may also contribute to plant defence.

One of the key follow-up steps for identification of important regulators of plant defence responses against HLB is to conduct functional screening to validate and characterize the candidate regulators identified from the comparative genomic studies. Functional genetic studies on tree crops, such as citrus, require significant time and effort due to the extended lifespan of a tree, low transformation efficiency and limited genetic resources (Fletcher *et al*., 2018). A rapid functional screening method has been lacking for genetic analysis of important genes identified from various genomic studies, including RNA-seq, sRNA-seq, MS/MS analysis. Recently, it was shown that *Nb* can be infected by CLas via transmission by the parasitic plant, dodder or Cuscuta (Pitino *et al*., 2018). *Nb* is considered the best plant host for genetic studies using VIGS to knock-down the gene of interest. CLso, which is vectored by the potato psyllid, can also infect *Nb* plants, but only through insect vector transmission. Although *Nb* is not an ideal host for potato psyllids, we identified the ideal conditions for the potato psyllid to infest *Nb*, which made it possible to utilize the *Nb*/Potato psyllid/*CLso* pathosystem as a model of the highly similar citrus/Asian Citrus psyllid/CLas pathosystem, for rapid functional screening of candidate defence regulators and pilot testing of new therapeutic compounds. Using this system, combined with gene knock-down studies in *Nb* using VIGS, we can study gene function and substantially reduce the time and cost involved in functional screening efforts. Subsequently, introducing the confirmed master regulators derived from disease-tolerant/resistant varieties, silencing the

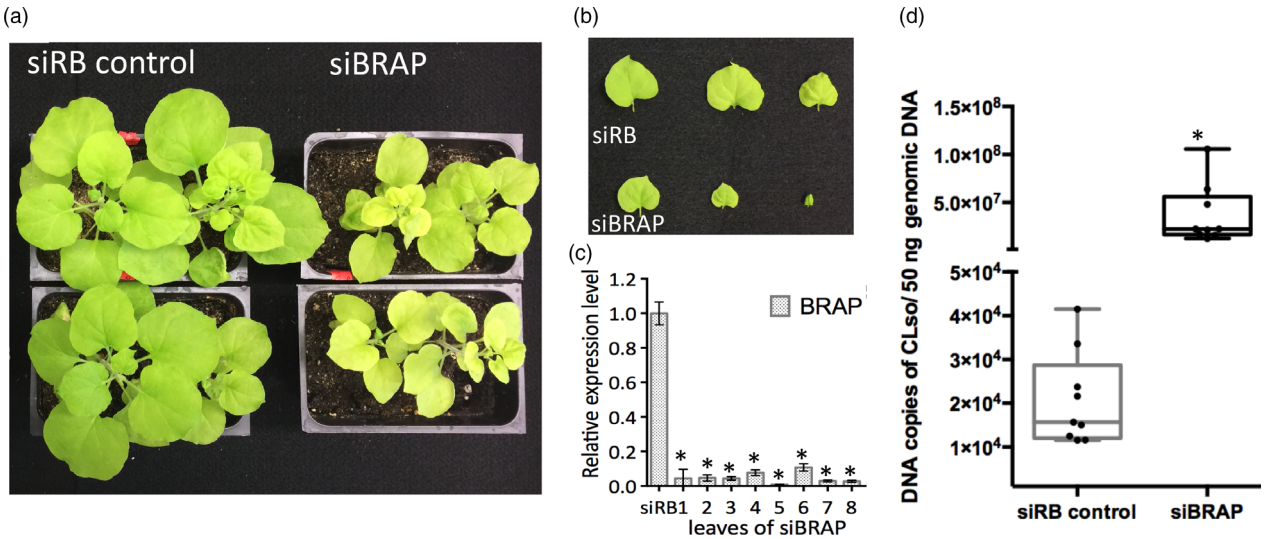

Figure 4 *Nb*/pysllid/CLso pathosystem in conjunction with VIGS reveals BRAP as a positive regulator of citrus immune response against CLso infection. (a) Two-week-old *Nb* plants were exposed to CLso-positive potato psyllids for 5 days before *BRAP* expression knock-down by VIGS. TRV vector carrying an unrelated potato gene RB (iRB control) was used as a control. (b) Details of leaves from panel a. (c) Expression level of *BRAP* in VIGS *Nb* plants analysed by qRT-PCR, normalized to the *Ubiqutin* gene (*NbUbi*). Two leaflets per plant were collected and four plants per treatment were used. Data are shown as means ± SE. *significant difference in comparison to the iRB control ($P < 0.01$; determined by ANOVA Dunnett's multiple comparisons test). (d) CLso bacteria titre measured by probe-based qPCR in 50 ng host genomic DNA. *indicates a significant difference ($P < 0.01$; determined by Student's *t*-test).

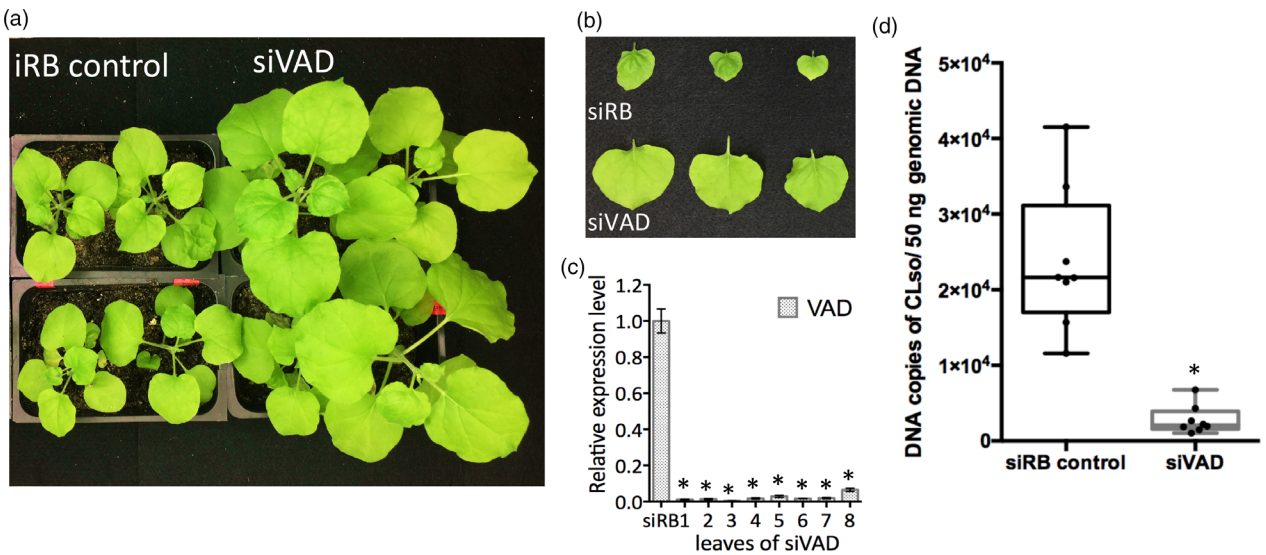

Figure 5 The *Nb*/pysllid/CLso pathosystem in conjunction with VIGS reveals VAD is a negative regulator of citrus immune response against CLso infection. (a) Two-week-old *Nb* plants were exposed to CLso-positive potato psyllids for 5 days before *VAD* expression knock-down by VIGS. TRV vector carrying an unrelated potato gene RB (iRB control) was used as a control. (b) Details of leaves from panel a. (c) Expression level of VAD in VIGS *Nb* plants was analysed by qRT-PCR, normalized to the *Ubiqutin* gene (*NbUbi*). Two leaflets per plant were collected and four plants per treatment were used. Data are shown as means ± SE. *Significant difference in comparison to the iRB control ($P < 0.01$; determined by ANOVA Dunnett's multiple comparisons test). (d) CLso bacteria titre measured by probe-based qPCR in 50 ng host genomic DNA. *a significant difference ($P < 0.01$; determined by Student's *t*-test).

negative regulators, or gene editing the commercially important varieties will help generate HLB-resistant or tolerant commercial cultivars and reduce the chance of genetic incompatibility. This approach allows us to discover with high confidence the potential master regulators that contribute to HLB tolerance and can assist in the development of new disease control therapies.

Experimental procedures

Plant material and CLas inoculation (US-942 and Cleopatra)

One-year-old greenhouse-grown Cleopatra mandarin (*Citrus reticulata*) and US-942 (*C. reticulata* x *Poncirus trifoliata*) seedlings

were used. Cleopatra is susceptible to CLas, while US-942 is tolerant (Albrecht and Bowman, 2012a). Plants were infected with CLas through grafting with buds from infected greenhouse-grown citrus plants. Control plants were mock-inoculated with buds from healthy disease-free citrus plants. Plants were cut back immediately after inoculation. For CLas detection DNA was extracted from 100 mg of ground leaf tissue using the Plant DNeasy Mini Kit (Qiagen) and real-time PCR assays were performed using primers HLBas and HLBr and probe HLBp (Li *et al*., 2006). For sRNA library construction, three healthy plants (control) and three CLas-infected plants of Cleopatra and US-942 with similar patterns of disease progression were used. Leaves and stems of plants were harvested 7 and 28 weeks post inoculation (wpi), immediately frozen in liquid nitrogen, and ground in liquid nitrogen using mortar and pestle. CLas inoculated Cleopatra plants were reduced in growth and displayed leaf chlorosis while CLas inoculated US-942 and mock-inoculated plants displayed no growth reductions or foliar disease symptoms.

Sydney hybrid 72&74

Sydney hybrid (*Microcitrus virgata*) is a hybrid of *Microcitrus australis* × *M. australasica* (Reuther *et al*., 1967). Seedlings of Sydney hybrid with different HLB disease responses were provided by Ramadugu. Syd72 was HLB-tolerant and Syd74 was susceptible in controlled greenhouse assays where the seedlings were exposed to CLas containing psyllids in a no-choice situation for two weeks. Symptom expression and CLas titre were monitored for an extended period of time and the disease response phenotype was determined.

Nb material, CLso inoculation and VIGS

Nb plants were grown in a plant growth room with 12-h light/12-h dark photoperiod at 23 ± 1 °C. 10-day-old *Nb* plants (with 2 true leaves emerged) were exposed to 10 CLso-positive nymphs of potato psyllid for five days. After three days recovery, VIGS was performed by infiltration with *A. tumefaciens* strain GV3101 containing the bipartite TRV, pTRV1 and pTRV2 vectors (Liu *et al*., 2002). Equal volumes of *A. tumefaciens* (OD_{600} = 0.5) suspensions containing pTRV1 and pTRV2-derived constructs or pTRV2-siRB non-target vector were mixed before infiltration (Song *et al*., 2003). The pTRV1- and pTRV2-carrying *A. tumefaciens* were then co-inoculated into the third and fourth true leaves of the plant by infiltration with 1ml syringe. The pTRV2-*siBRAP and pTRV2-siVAD* construct which can silence *NbBRAP* and *NbBVAD*, respectively, was designed by the SGN VIGS Tool (Fernandez-Pozo *et al*., 2015) and the primer sets used for amplifying the fragment are shown in Table S10. The leaves of inoculated plants were collected after three weeks for DNA and RNA extraction.

RNA preparation and qRT-PCR

Citrus Total RNA was extracted from ground tissue using acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski et al., 1987), followed by phenol/chloroform/isoamylalcohol extraction and precipitation with isopropanol.

Nb total RNA was extracted using TRIzol Reagent (Invitrogen) and treated with DNase I (Roche). Total RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen) with small RNA specific RT primers or oligo(dT) primer (Table S10). For qPCR, transcripts were amplified from 2 μL of a 20 × diluted cDNA and iQ SYBR Green Supermix (Bio-Rad). The PCR amplification consisted of 3 min at 94 °C, 45 cycles of 30 s at 94 °C, 30 s at 62 °C and 1 min at 72 °C, 15 min at 72 °C, followed by the generation of a dissociation curve. The generated threshold cycle (CT) was used to calculate the transcript abundance relative to *Nb Ubiquitin* (*NbUbi*) (Jin *et al*., 2002) or Citrus *Actin* (*CsAct*)(Zhao *et al*., 2013)(Table S10).

Small RNA library construction and sequence analysis

Total RNA was resolved by a denaturing 14% polyacrylamide gel, from which 18–28 nt RNA fragments were recovered. The purified fragments were ligated to a 5′ adaptor (GUUCAGAGUUCUACAGUCCGACGAUCAG), and a 3′ adaptor (UCGUAUGCCGUCUUCUGCUUG) according to the Illumina small RNA-seq library prep kit protocol. The ligated fragments were gel-purified and reverse-transcribed by SuperScript III (Invitrogen, Carlsbad, CA, US). After PCR amplification for 15 cycles with Phusion High-Fidelity DNA Polymerase (ThermoFisher, Waltham, Ma, US), the PCR products were gel-purified and sequenced according to the Illumina sRNA sequencing protocol. Total mRNA or sRNA-enriched preparations were used to generate cDNA fragment libraries with the respective Illumina kits for the Illumina HiSeq-2000 platform. The adaptor-trimmed and quality-filtered sRNAs were clustered based on their mapping positions. The number of reads normalized to total reads was used to estimate their expression. Conserved miRNAs were identified by sequence similarity searches against the registry of plant microRNAs in miRBase (Kozomara and Griffiths-Jones, 2014). The targets of conserved and novel citrus miRNAs or siRNAs were then predicted by psRNATarget against the transcript library of Citrus clementine (JGI genomic project, Phytozome 11 182 v1.0; Dai *et al*., 2018). DAVID bioinformatics resources was used for the Gene Ontology (GO) enrichment analysis of the target genes (Huang et al., 2009a, 2009b).

CLso detection by real-time PCR

To detect CLso in Nb plants, plant DNA was purified following the CTAB (cetyltrimethyl ammonium bromide) protocol from the mid-vein of leaves (Murray and Thompson, 1980). Then, CLso was detected by quantitative real-time PCR in the plant using the primers, TaqMan probe designed against *CLso* 16S rDNA (Table S10). The primers and probe of the positive internal control set were designed on basis of the ubiquitin gene from *Nb* (*NbUbi*). The amplification protocol of quantitative PCR was set at 40 cycles with 95 °C for 10 min and 60 °C for 30 min. Dilutions of plasmid DNA pLso (containing target DNA sequence from CLso in pGEM-T easy vector, Promega) were used to set a standard curve for evaluating the titre of CLas.

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Conflict of interest

None of the co-authors have a conflict of interest to declare.

Authors' contribution

H.J. conceived the original idea and supervised the whole project. H.J. and C.H. designed the experiment. C.H., D.N., G.K., M.J.,

L.N. and C.B. carried out the experiment. C.R. provide the citrus material. C.H. wrote the manuscript with support from H.J., J.T., U.A., C.R. and K.B. U.A., K. B.. All authors read and approved of its content.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. The GO enrichment analysis of US-942 small RNA target genes reveals that regulators involved in defence responses may contribute to the HLB-tolerance of US-942.

Figure S2. HLB-tolerant Syd72 has higher expression level of miR399 than HLB-susceptible Syd74.

Figure S3. The expression pattern of four selected sRNAs (Figure 2) in infected Cleopatra and US-942 plants was measured at 7 and 28 weeks post inoculation (wpi).

Figure S4. The expression pattern of the target genes of the selected sRNA regulators (Figure 2) was measured in infected Cleopatra and US-942 plants at 7 and 28 wpi.

Figure S5. Disease development of Nb plants was shown at two weeks after infection with *C*Lso by potato psyllid nymph transmission.

Table S1. miRNAs that are differentially expressed in US-942 and Cleopatra.

Table S2. Small RNAs that have higher constitutive expression levels in US-942 compared to Cleopatra.

Table S3. Predicted target genes of sRNAs in Table S2.

Table S4. Small RNAs that have lower constitutive expression levels in US-942 compared to Cleopatra.

Table S5. Predicted target genes of Small RNAs in Table S4.

Table S6. Small RNAs up-regulated after CLas infection in US-942 but not in Cleopatra.

Table S7. Small RNAs down-regulated after CLas infection in US-942 but not in Cleopatra.

Table S8. Predicted target genes of the top 50 small RNAs in Table S6.

Table S9. Predicted target genes of the top 50 small RNAs in Table S7.

Table S10. Primers used in this study.

